AD NUMBER

AD840030

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

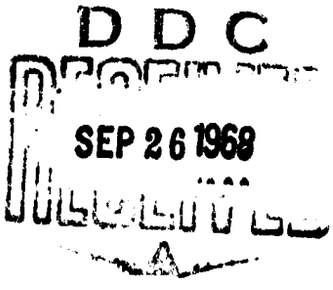
Distribution authorized to U.S. Gov't. agencies and their contractors; Foreign Government Information; FEB 1968. Other requests shall be referred to Department of the Army, Fort Detrick, Attn: Technical Release Branch/TID, Frederick, MD 21701.

AUTHORITY

SMUFD D/A ltr, 15 Feb 1972

TRANSLATION NO. 324

DATE: 16 February 1968



DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

2134

FLUORESCENT ANTIBODIES IN DIAGNOSIS AND RESEARCH

K. D. Jentzsh*

Monatshefte fur Veterinarmedizin (Monthly Periodical of Veterinary Medicine)
Vol 18, No 1, 15 February 1963, pages 123-128

The endeavors of medicine, to establish an exact diagnosis in the shortest possible time, follow the ethical principle of this profession to help the patient as fast as possible. A quick and exact diagnosis plays a decisive role in the choice of therapy and it is also the best demonstration of the physician's ability.

It is self-evident that disciplines which contribute to the clinical diagnosis with their auxiliary tests, as for example bacteriology, are subject to the same requirements with respect to exactness and speed. It should always be the main objective of the bacteriologist to make a well-considered and purposeful selection of the most modern available bacteriological procedures in order to effect the breeding and identification of isolated germs by the fastest route. On this maneuverability depend, to a large degree, the clinician's prospects for success. Bacteriology has met these requirements by constantly adopting new cultural and serological procedures and, thus, considerably reducing the duration of the tests.

In recent times, the bacteriologist is offered a new process which, at least in certain cases, promises a significant reduction in the test time and which, because of

*Author: Dr. K. D. Jentzsh, Berlin-Friedrichsfelde, Wilhelmstrasse 4.

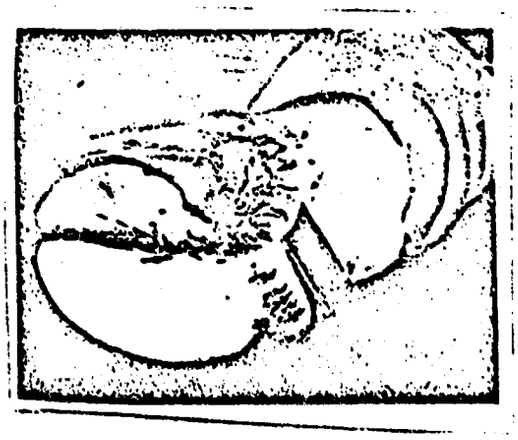
its immunological specificity, is worthy of a thorough investigation and practical application. Ever since the successful work of Coons and Kaplan (1950) concerning the binding of stains to immunoglobulins, the "Fluorescent Antibodies" (FA) have become a firm concept in world literature. Anglo-American literature, especially, contains extensive treatises which evaluate the process positively. Within its immunobiological limits, the process brought good results, while the traditional methods were not able to achieve similar results with equal economy of time and equipment. The FA (Fluorescent Antibodies) have a high degree of immunological specificity and, thus, they fulfil the chief prerequisite for application in bacteriological practice.

The FA are immunoglobulins that are tagged with fluorescent stains [7"fluorochromes" according to Haitinger (1938)7 and they serve as a reagent for the proof of homolog antigens in smears or tissue specimens. A comprehensive description of the manufacture and of the techniques of application is outside the scope of this paper and, therefore, we should like to mention only what is necessary for the understanding of the text.

The FA are manufactured by a chemical precipitation of gamma globulins that contain antibodies and that are obtained from immune sera. The excess precipitant, usually ammonium sulfate, is removed through dialysis. The globulin solution is purified, and a suitable fluorochrome is added which combines with the serum globulins to form a protein-stain complex. At first, fluorescein-isocyanate of Coons and Kaplan (1950) was used as the indicator stain. The drawback of this stain was its short durability. Today, one uses chiefly fluorescein-isothiocyanate [Riggs, Seiwald, Burckhalter, Downs, and Metcalf (1958)7 which is a relatively durable yellow-orange powder. However, since this compound is not manufactured commercially, it has to be self-made. Its synthesis is tedious and requires chemical knowledge and special equipment; furthermore, it has the tendency to form nonspecific precipitates in histological tissue specimens. Contrary to this, 1-dimethylaminonaphthaline-5-sulfochloride, introduced by Clayton (1954), Mayersbach (1958), and Redetzki (1958), apparently does not possess the latter disadvantage and is easier to synthesize. A measured amount of this compound is added to the globulin solution and is allowed to combine with the globulin. The excess stain is removed through dialysis, and a check with homolog antigens is performed. Then the FA

solution is suitably diluted and either frozen or preserved in another way.

The working principle of the FA is as follows. When a prepared smear or tissue specimen containing bacteria is treated with the homolog FA, an antigen-antibody reaction takes place between the bacteria and the antibodies. The tagged antibodies combine with the homolog bacteria in such a way that they cannot be removed any more from the bacteria through subsequent washing. On the other hand, the free (not bound) FA-tagged antibodies can be easily and completely rinsed away. During the examination of the dried and sealed slide in short-wave ultraviolet or blue light, the fluorochrome particles remaining with the antibodies are stimulated to emit long-wave color light, that is, they fluoresce. The fluorochromes mentioned above give a yellow-green fluorescence. Since the fluorochromes are bound through the antibodies to the bacteria, the form of the bacteria appears as a microfluorescent picture (Fig. 1).



GRAPHIC NOT REPRODUCIBLE

Figure 1. Direct proof

Legend: 1. Antigen 2. Antibody
3. Fluorochrome 4. Fluorescent antibody
5. Fluorescent antigen-antibody complex.

GRAPHIC NOT REPRODUCIBL

This arrangement of the test is called the "direct proof" [Coons and Kaplan (1950)].

The "indirect proof" or the "sandwich method" of Weller and Coons (1954) works essentially on the same principle (Fig. 2). In this method the antibody is not tagged, so that at first a nonfluorescent antigen-antibody complex is formed that is not visible fluoromicroscopically. The complex is made visible through a subsequent overlaying with a tagged, precipitating serum. The serum precipitins are directed against the antibodies and, for this reason, they are called anti-antibodies. At the end, a fluorescent antigen-antibody-anti-antibody complex is formed.

Figure 2. Indirect proof

Legend:
1. Antigen
2. Antibody
3. Anti-antibody (antiglobulin serum)
4. Fluorochrome
5. Antigen-antibody complex
6. Fluorescent anti-antibody
7. Fluorescent antigen-antibody-anti-antibody complex.

GRAPHIC NOT REPRODUCIBLE

The following example may serve as a better explanation. When anthrax bacilli are to be identified in a smear, the smear is treated with a non-tagged anthrax bacilli antibodies obtained, e.g., from the immune serum of rabbits. A nonfluorescent complex of anthrax bacilli and rabbit antibodies is formed. Subsequently, the slide is covered with a tagged anti-rabbit-globulin serum obtained through immunization of an animal of a different but suitable species. This leads to a fixation of the fluorescent anti-rabbit-globulin antibodies to the already existing complex of anthrax bacilli and rabbit antibodies. After the excess fluorescent anti-rabbit globulins are washed away, the complex shines characteristically in the fluoromicroscope.

In our example, the "indirect proof" has the advantage that the anti-rabbit-globulin serum can be used over and over again for all systems in which rabbit antibodies are used [Carter and Leise (1957)]. Furthermore, this method gives a more intense fluorescence resulting from an increased surface area. After the antibody has been bound to the antigen, a much greater number of fluorescent anti-antibodies can be bound by a single bacteria cell because the antibodies assume the role of antigens in relation to the anti-antibodies, and because one antigen molecule always binds several antibody molecules. This relation is illustrated to a certain degree in Fig. 2 which shows an increase in the surface area of the antigen-antibody-anti-antibody complex. The "indirect proof" is suitable for determinations of antibodies in blood serum and in histological tissue specimens (see below).

The fluoro-immunological demonstration of bacteria offers significant advantages for the diagnosis. A very important asset for the practical application is the specificity of the process which, however, must be thoroughly checked for each antigen.

The specificity of the FA technique lies within the immunobiological limits applying to all serological methods, that is, the procedure will not work in cases where a specific immunological reaction between the antigen and the antibody is not to be expected. Even when the applicability is warranted and considered suitable for routine procedures, every test should be accompanied by positive and negative controls that would demonstrate beyond doubts that the fluorescence is caused by a specific reaction. In this way false diagnoses can be avoided. Group antigens lead

to overlapping reactions. For this reason, the differentiation of bacteria types with FA is possible only with carefully saturated sera, as has been shown with the polysaccharides of pneumococci and Friedlander bacteria [Kaplan, Coons, and Deane (1950), Hill, Deane, and Coons (1950)]. In the diagnosis of salmonella with FA of polyvalent immune sera, cross reactions occur quite frequently when the tests are done directly on stool specimens; thus, the application of the FA to this routine procedure is not feasible [Thomason, Cherry, and Edwards (1959)]. The tagged polyvalent serum stains all salmonella strains without exceptions. In addition, related arizona, coli, proteus, and paracoli intermedius strains also enter the reaction, while the shigella give no cross reactions. The antigen kinship within the family of enterobacteriaceae is well known and it occasionally presents difficulties in the traditional serological differentiation. On the other hand, the demonstration of enteropathogenic coli germs in children stool specimens is completely successful due to the uniform composition of the bacterial flora [Whitaker, Page, Stulberg, and Zuelzer (1958)].

Because of common antigens, cross reactions occur between malleomyces mallei and m. pseudomallei and, therefore, a fluoro-immunological differentiation of these two is not possible [Moody, Goldman, and Thomason (1956)]. Similarly, common reactions occur between streptococci of various groups that can be differentiated through precipitation [Moody, Ellis, and Updyke (1958), Halperen, Donaldson, and Sulking (1958)]. Nevertheless, the utilization of the FA is warranted in special cases, as for example in the analysis of throat smears [Halperen, Donaldson, and Sulkin (1958)]. For the same reason, one can not differentiate fluoro-immunologically between bacillus anthracis and bac. cereus [Cherry and Freeman (1959)]. True cross reactions occur also between anti-brucella FA and the bact. tularensis [Moody, Siegeleisen, and Taylor (1961)]. However, the specificity of the FA is safeguarded by the fact that cross reactions between nonrelated species of bacterial strains take place only exceptionally because a large number of heterology bacteria species do not react with any but the specific FA. Nevertheless, these exceptions have to be determined and constantly kept in mind.

The serological affinity reactions occur in the FA technique because this procedure possesses an extraordinarily high diagnostic sensitivity. This has been confirmed

without reservations by all authors. While the traditional examination of clinical material or of organ parts requires a cultural breeding or even an enrichment of the bacteria, the proof of germs with the FA method is successful on a smear or a cast directly on the initial material.

For this purpose, the fixed histological specimen is simply covered with a few drops of the FA solution and kept for approximately 30 minutes in a humid room at incubator temperature to permit the binding of the FA to the antigens. As soon as the not bound FA is rinsed away and the slide is air-dried and sealed, the fluoro-microscopic examination can proceed.

It is possible to judge with certainty about a single germ (!) whether it is homolog to the applied FA. This sensitivity is unique and has not been achieved as yet with the other methods. Admittedly, our procedure has a practical value only in cases when there is a hint about the type of the presumably present germs (e.g., through section) or when a limited selection of routine examinations are performed aiming to establish either the presence or the absence of a specific germ. In such cases (not to mention the required controls) the tissue slides have to be treated only with the applicable FA. It would be erroneous to attempt to replace entirely the bacteriological examination of organs with the FA technique. For this purpose, an enormous number of various FA would be necessary, and the method would lose its advantages with respect to simplicity and time economy. Therefore, we are thinking primarily of the routine tests performed in clinical laboratories, as for example, identification of brucella in secreta, exudates, rinse samples, egg skins, and dead embryos; investigations of milk sediments for brucella, streptococci, and other germs; tests for anthrax bacilli in suspected organ specimens or blood; investigation of sputum throat smears, and others for definite germs, etc. Of special significance is the fact that, when culture investigation becomes impossible because of contamination with secondary germs or decay bacteria (e.g., overgrowth of petri dish cultures with proteus), the specific proof of the germs with the FA is generally still guaranteed. In many cases, the animal test becomes superfluous.

Especially promising fields of application are to be expected with pathogenic germs which are difficult to grow and whose growth takes a long time, e.g., clostridia, sphaerophorus (necrosis bacteria), actinomycetae, etc. The

diagnosis would be considerably sped up. However, preliminary investigations must establish whether or not, and to what degree specific FA can be made for the respective types of germs. It is possible that the FA technique can be extended to the diagnosis of tuberculosis bacteria. We have already begun studies in this direction. Success can also be expected in the diagnosis of toxoplasmosis [Goldman (1957)] and leptospirae [Moulton and Haworth (1957)]. Of further importance is the evidence that, under suitable conditions, also virus diseases can be diagnosed by means of the FA. The inclusion bodies in rabies [Goldwasser and Kissling (1958), Topleninova and Remezov (1960)], of distemper [Moulton and Browns (1954), Coffin and Liu (1957)], and of hepatitis contagiosa canis [Coffin, Coons, and Cabasso (1953)] were demonstrated fluoro-microscopically. Similarly, the proof of smallpox virus [Noyes and Watson (1955)], of psittacosis virus [Buckley, Whitney, and Rapp (1955)], and of poliomyelitis virus [Buckley (1957)] in tissue cultures are successful.

The degree of sensitivity of the FA technique can be defined exactly. The limit of a sure antigen proof can be established theoretically by the case where the antigen distribution in the initial material is so rare that there is no certainty any more that even a single minimal unit of antigen particles will be transferred with the loop or through casting onto the slide to be analyzed. The minimal number of malleomyceae mallei in saline amounts to 220 germs per ml. The identification of m. pseudomallei through agglutination requires, on the other hand, 10^8 germs per ml [Thomason, Moody, and Goldman (1956)] A similar relation applies to the brucellae [Moody, Biegeleisen, and Taylor (1961)]. In a pure culture, brucella can be diagnosed with certainty at a concentration of 2.5×10^3 germs per ml. The simultaneous presence of 6.8×10^5 serratia germs per one brucella germ (!) does not disturb the specific proof of the brucella. However, approximately 6×10^7 brucellae per ml are required for a positive agglutination. According to the newest data [Biegeleisen, Moody, Narous, and Flynt (1962)], the identification is considered reliable when at least 2×10^2 brucellae are present in each ml of the suspension. If we consider that for the preparation of the smear only a few loops of the initial medium are necessary and that the number of transferred germs is correspondingly small, we begin to understand the sensitivity of the procedure. Hobson and Mann (1957) reported equally good results in the differentiation of rumen bacteria.

Next to the exactness, the FA technique offers also the advantage of a significantly shortened investigation time. While the culture or serological investigation requires at least 24 to 48 hours, the FA determination of germs in a smear or an imprint is possible, on the average, after one hour. Moody, Biegeleisen, and Taylor (1961) quote 96 hours for the diagnosis of brucella in a culture with the traditional method and only one hour for the FA method. They investigated 10 artificial brucella preparations in organic material and were able to establish positive diagnoses in six cases with the FA and in only three cases with the other methods. Further study concerning the practical application of the FA for brucella diagnosis was also successful [Biegeleisen, Moody, Marcus, and Flynt (1962)].

The reduction in the test time with the FA becomes more apparent the longer is the time required for the differentiation of the germs with the traditional methods. For this reason, we recommend to investigate those bacteria for FA feasibility (as has been mentioned in connection with the sensitivity of the FA technique) which set high demands on the nutrient medium and require an extraordinary long period of time for growth and differentiation.

The advantages which the FA offer to the clinical diagnosis apply equally well to research work. The FA technique may become very useful in this branch of science. For example, it may be worthwhile to investigate bacteria types for which the utilization of the FA in the clinical praxis is not an absolute necessity because they occur very rarely. Thus, one may investigate the spread of certain germs in specimens of organs, earth, fodder, or water. The FA approach could be more successful than the culture method.

At this point we should like to mention that dead bacteria can be specifically diagnosed with the same exactness. Up till now, no other bacteriological method could claim this diagnostic finesse! The great accuracy of the FA would be especially valuable in epidemiological investigations. Owing to the ability of the FA to detect the presence of dead bacteria, many fields can be re-examined. Petuely and Lindner (1959) succeeded in analyzing quantitatively all the germs that constitute the intestinal flora. Prior to that time, it was possible to cultivate not more than 1-2% of the germs found in the intestinal content. The remaining 98-99% were regarded as dead or not suitable

for breeding with the available methods.

In experimental work on animals, the utilization of the FA on tissue sections (obtained with minimal harm to the animals) offers a far-reaching insight into the distribution of bacteria and other antigens in the organism. With the help of the FA, it is possible to perform a detailed study of the spread of the antigens through the tissues and through individual cells. For example, injected albumins and globulins were demonstrated with the FA not only in the cytoplasm of the cells of the reticulo-histocytic system but partially also in the nuclei of these cells [Coons, Leduc, and Kaplan (1051)]. Investigations of the brucella spread in the bodies of experimental animals which had died of brucellosis, demonstrated the presence of brucella antigens not only in the form of bacteria but also in the form of intra- and extracellularly dissolved amorphous masses [Biegeleisen, Moody, Marcus, and Flynt (1962)].

Approaching the problem from the opposite end, the FA can be used for the study of the sites of antibody formation in the various tissues of the organism [Coons, Leduc, and Connolly (1955)]. It is possible, by means of the "indirect proof" in tissue specimens of experimental animals treated with globulins or albumins, to demonstrate that plasma cells function as producers of the antibodies. The present concepts of the formation of antibodies could be underpinned and broadened [Leduc, Coons, and Connolly (1955)].

We could cite numerous animal experiments in which a specific proof of antigens and their spread in the organism would yield considerable contributions to the clarification of controversial problems. As a general example, we should like to point out that through the fluoro-immunological proof of listeria antigens in the trigeminal nerve -- considered the route of infection in the cerebral listeriosis of sheep -- a comprehensive evaluation of the histological changes in this nerve could be performed. Histopathological methods, even with the utilization of culture procedures, would make such an evaluation possible only under unusually favorable conditions. The FA method is especially recommended here because the experimental production of sheep listeriosis is not always successful and, thus, the animal experiments are not reliable. With carefully prepared modes of application, the FA method can contribute to the clarification of the pathogenesis of many infectious diseases because it lends specificity to

the histological changes and, thus, facilitates the interpretation of various cellular reactions.

The cited advantages and the apparently easy production and application of the FA should not obscure the fact that the FA technique is still far remote from a general practical application. The difficulties are of a technical nature and they are caused chiefly by the inadequate supply of suitable fluorochromes. It is possible to synthesize the dyes in one's own chemical laboratory following exactly the formulas available in the literature. However, this would mean a multiple dissipation of work and material expenditures. This annoyance could be avoided by establishing a central manufacturing plant. Unfortunately, at present neither the demand nor the need are sufficiently high to make a central manufacture of the FA worthwhile. This vicious circle could be broken if the FA technique were worked out to perfection for certain specific and feasible fields of application and then were generally recommended to bacteriological laboratories. With a sufficiently high demand, it would even be possible for the central plant to manufacture and keep in stock for sale labeled immune sera and antiglobulin sera. This is done successfully in the DIFCO Laboratories in Michigan, U.S.A. Such arrangement would remove a great burden from the test laboratories, and the advantages of the FA technique would come to full light.

Conclusion

The advantages resulting from the application of fluorescent antibodies to diagnosis and research are discussed. By means of examples and suggestions, it is explained that in individual cases the procedure is quite suitable for practical diagnosis and that it can be successfully utilized in research. In order for the method to be useful, it has to be restricted to the most promising fields, and the test laboratories have to be supplied with completely worked-out procedures and with suitably and clearly labeled immune sera. If these prerequisites are fulfilled, the method will save time and material and, thus, serve the needs of practical work.

We should like to express our thanks to Mrs. R. Richter, our scientific draftswoman.

BIBLIOGRAPHY

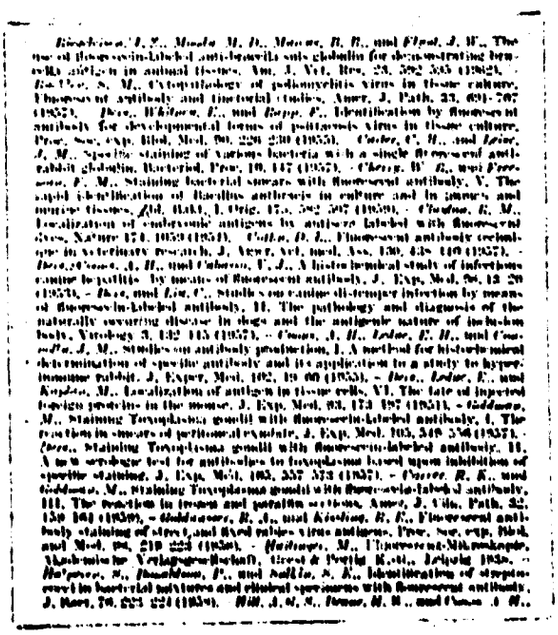
Haitinger, M. "Fluorescence-Microscopy", Akademische Verlagsgesellschaft (Academic Press), Geest & Portig K. -G., Leipzig, 1938.

Mayersbach, H., "Immunohistological Methods, II. Ein weiterer Markierungsfarbstoff: 1-Dimethylnaphthylaminsulfonsaure-5" (A further Indicator Stain: 5-dimethylamino-1-naphthelane sulfonyl acid), Acta histochemica (Histochemical Proceedings), Vol 5, 1958, pages 351-368.

Petuley, F., and Lindner, G., "The Method of Analyzing the Intestinal Flora with Fluorescent Antibodies (Fluorescent taggin with 5-dimethylamino-1-naphthelane sulfonyl acid," Schweiz. Z. Path. Bakt. (Swiss Journal of Pathological Bacteriology), Vol 22, 1959, pages 747-751.

Topleninova, K. A., and Remezov, P. I., "Contributions to the Diagnosis of Rabies," Veterinariya (Veterinary Medicine), Moscow, Vol 11, 1960, pages 85-88.

The other references are in English.



GRAPHIC NOT REPRODUCIBLE